IN VITRO INHIBITORY ACTIVITY OF TRICHORZIANINES ON *SCLEROTIUM ROLFSII* SACC.

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ABSTRACT - Trichorzianines A and B obtained from *Trichoderma harzianum* were tested on the mycelial growth of *Sclerotium rolfsii*. Bioassays evidenced that the trichorzianines had different inhibitory activity. These metabolites produced a change in the morphogenetic pattern of mycelia. Bioassays also showed that trichorzianines A inhibited the own mycelia of *T. harzianum*, which could be considered as an absence of immunity. Effects of trichorzianines on plasma membrane are discussed.

RÉSUMÉ - Les trichorzianines A et B obtenues à partir de cultures de *T. harzianum* ont été testées pour leur activité antagoniste sur le développement mycélien de *S. rolfsii*. Les résultats expérimentaux montrent que les deux types de trichorzianines ont des activités différentes sur la croissance et la morphogenèse mycéliennes de *S. rolfsii*. Les essais effectués mettent aussi en évidence l'absence d'auto-immunité de *T. harzianum* vis à vis de ses propres trichorzianines. L'effet probable de ces dernières sur les membranes cellulaires est discuté.

KEYWORDS - *Sclerotium rolfsii*, *Trichoderma harzianum*, growth inhibition, trichorzianines, self-inhibition.

INTRODUCTION

Several studies about antibiotic production by fungal strains and their effect on different plant pathogens were performed under laboratory conditions being *T. harzianum* one of the most active (Fravel, 1988; Ghisalberti *et al.*, 1990; Scarselletti & Faull, 1994). However, very little is known about the antibiotic activity of *T. harzianum* on mycelial growth of the important soilborne plant pathogen *Sclerotium rolfsii* (Bell *et al.*, 1982). Three categories of antibiotics produced by *T. harzianum* (and other *Trichoderma* species) can be recognized: "volatiles", e.g. 6-pentyl-α-pyrone [6-p-p] and most of the isocyanide class of compounds; "leachables", materials with some solubility in water and "peptaibols", which consist of hydrophobic peptides (Ghisalberti & Sivasithamparam, 1991). These last compounds are peptides with 7 to 20 aminoacid residues, containing a high proportion of α-aminoisobutyric acid, with acetylated N-terminal and C-terminal aminoalcohol (Bodo *et al.*, 1985; El Hajji *et al.*, 1987, 1989).

Trichorzianines are a mixture of molecules of the peptaibol class produced by *T. harzianum* which, interact with lipid membranes and modify their permeability (El Hajji *et al.*, 1989).

TA are linear neutral monodecapeptide with an acetyleted N terminal residue and a C terminal amino alcohol. TB are the acidic anlogues due to the replacement of a glutamine at position 18 in the sequence by a glutamic acid.

The aim of this work was to evidence a causal link between trichorzianines produced by *T. harzianum* and their *in vitro* inhibitory activity on the mycelial growth of *S. rolfsii* as well as on the *T. harzianum* mycelium itself.

MATERIALS AND METHODS

Fungal strains

Trichoderma harzianum Rifai (MVHC 6063) was used for production of trichorzianines and *Sclerotium rolfsii* Sacc. (MVHC 5407) was the plant pathogen used in this study. Each fungus was subcultured on 2% malt-agar (MA) and grown at 24°C in the dark and the strains were preserved on 2% MA slopes at 5°C.

Extraction of trichorzianines

Fungal cultures were performed by inoculating 200 ml of synthetic media with 1 ml of spores suspension in 1 l Roux flasks. The synthetic medium was composed of: 5 g glucose; 0.8 g KH_2PO_4; 0.7 g KNO_3; 0.2 g $CaHPO_4$; 0.5 g $MgSO_4$; 10 mg $MnSO_4$; 10 mg $ZnSO_4$; 5 mg $CuSO_4$; 1 mg $FeSO_4$; in 1 l distilled water pH 6. Sixty flasks of stationary culture were incubated at 24°C until sporulation. Extraction of trichorzianines was performed according to the method proposed by Rebuffat *et al.* (1991).

Each liquid culture of *T. harzianum* was filtered in a Büchner funnel to separate mycelium from culture broth. Wet mycelium was extracted three times with methanol at room temperature and the extracts combined and evaporated to dryness. The filtered broth was extracted three times with n-butanol, the extracts were then combined and evaporated to dryness. Both mycelial and broth extracts were treated with hexane using the same procedure as for methanol or n-butanol. The insoluble broth fraction obtained was initially fractionated by Sephadex LH-20 chromatography (MeOH), then on silica-gel column (SiO_2, Merck; CH_2Cl_2/MeOH 80:20-50:50). Thin-layer chromatograms (TLC) were performed on all kinds of extracts using a SiO_2 plate (Polygram Sil G/UV); CH_2Cl_2/MeOH 80:20, and visualized by spraying with anisaldehyde/H_2SO_4/acetic acid (1:0.5:25) reagent or H_2SO_4 10% (v/v). Trichorzianines yet obtained by the co-authors were used as control for the TLC.

Bioassays

The different fractions obtained were tested for their antagonistic activity against the mycelial growth of *S. rolfsii*. A methanol solution of each fraction was

mixed with 2% liquid MA (45-50°C) and 2.5 ml were poured in small Petri dishes (5 cm diameter), which were then inoculated with a fresh sclerotium (14 days old) and incubated at 24°C. The inhibitory activity of the peptide mixture was tested with 25, 50 and 100 $\mu g.ml^{-1}$ of MA medium and, the trichorzianines A and B at 100 $\mu g.ml^{-1}$. Four sets of concentrations with 0, 2, 4 and 10 $\mu l.ml^{-1}$ of methanol in 2% MA were prepared as controls to evaluate the incidence of methanol on mycelial growth. Treatments and controls were replicated five times. The diameter of the *S. rolfsii* colonies was measured and the percentages inhibition of growth was calculated from mean values at 72 h as follows: 100-(dt.100/DT), where dt is the diameter of the treated colony and DT is the diameter of the control. Differences between the inhibitory activity of each *T. harzianum* extract on the *S. rolfsii* mycelium were evidenced by means of ANOVA (Service des Etudes Statistiques, Institut Technique des Céréales et des Fourrages, France, STAT-ITCF). Mycelial growth was observed until 12 days.

The activity of trichorzianines A on mycelial growth of *T.harzianum* was tested and dry weigth of mycelium was calculated. The inhibitory activity was calculated as previously described for *S. rolfsii*.

RESULTS

The soluble hexane fraction from *T. harzianum* mycelia and broth had not affected the mycelial growth of *S. rolfsii* as well as insoluble hexane fraction extracted from mycelium (P>95%). Conversely, the insoluble hexane fraction extracted from broth inhibited the mycelial growth. When this extract was fractionated on Sephadex LH 20 a peptide mixture was obtained. The activity of this fraction showed that the inhibitory activity increased up to 80% at a concentration of 100 $\mu g.ml^{-1}$ (P>99%, Plate 1). The peptide mixture fractionated on silica gel resulted in two groups of trichorzianines termed A (TA) and B (TB). TA inhibited 70% (P>99%) and TB 36% (P>95%) of the *Sclerotium* mycelial growth (Table 1).

Table 1. Inhibition of *S. rolfsii* mycelial growth after 72 hours (in percent, GI%) by mycelium, mixture and different peptides fractions. Values follow by a differ at probability (P)>95%, *b* at P>99% as determined by ANOVA and *c* do not differ.

Tableau 1. Activité inhibitrice du mycelium, du mélange et des différentes fractions peptidiques sur la croissance de *S. rolfsii* après 72 heures de culture (en % par rapport au témoin). Les valeurs suivies de *a* diffèrent avec une probabilité (P)> à 95%, *b*> 99% et *c* n'est pas différente du témoin.

Products	Concentration of products ($\mu g.ml^{-1}$)	GI%
Mycelium fraction	$2x10^3$	-9 *a*
Mixture of peptides:	25	11 *c*
	50	56 *a*
	100	80 *b*
Trichorzianines A	100	70 *b*
Trichorzianines B	100	36 *a*

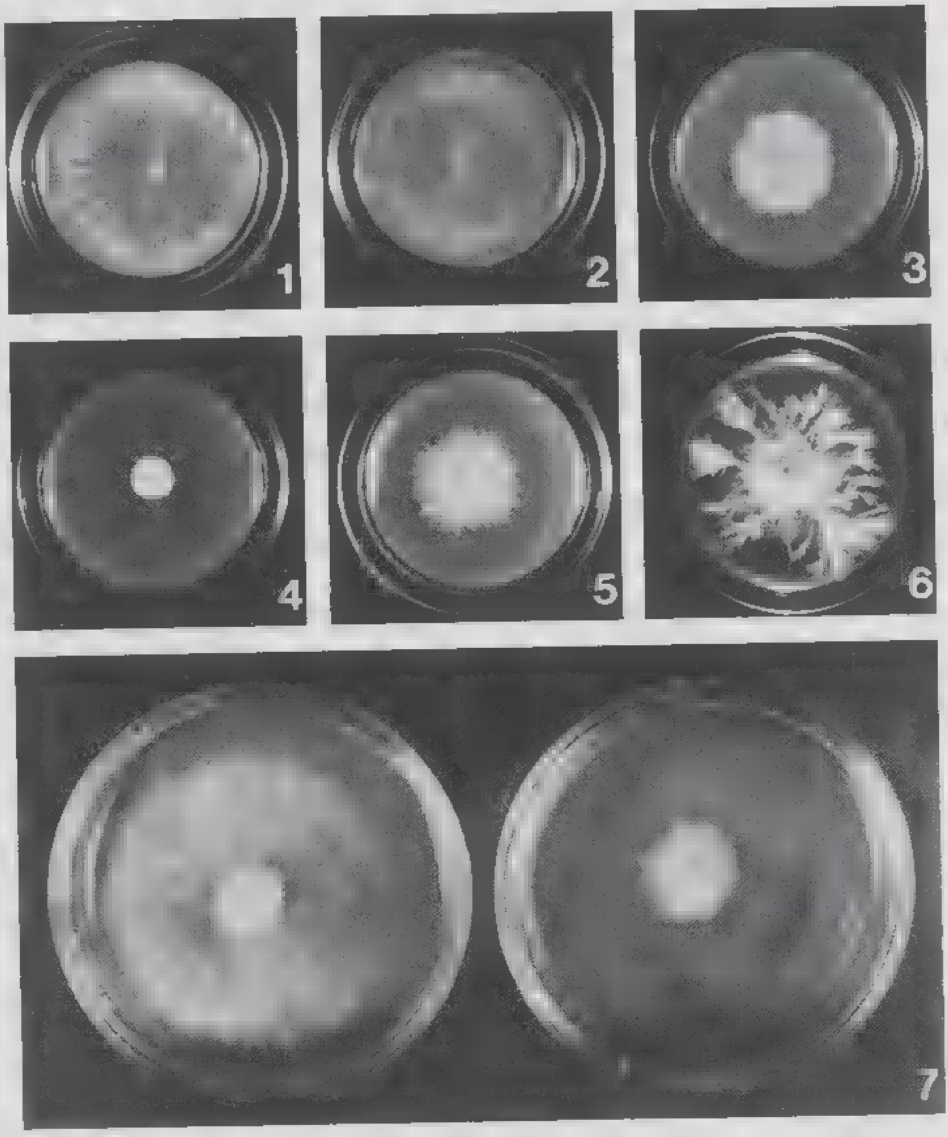

Plate 1. Peptide growth inhibition of *S. rolfsii* after 96 hours. 1: *S. rolfsii* control growing on MA medium; 2-4: mixture of peptides at different concentrations (25, 50, 100 $\mu g.ml^{-1}$). 5: Effect of low molecular weight fraction. 6: Effect of peptide mixture (100 $\mu g.ml^{-1}$) on growth pattern of *S. rolfsii* after 10 days. Strands of mycelium ▬ opening up in ■ fanlike fashion. 7: Trichorzianines A growth inhibition of *S. rolfsii* after 96 hours. Left: *S. rolfsii* control growing on MA medium; right: with TA (100 $\mu g.ml^{-1}$).

Growth was limited but never completely stoped and after 10 days the mixture of peptides induced change of growth pattern of *S. rolfsii* at 100 $\mu g.ml^{-1}$. Strands of aerial mycelium grew out, opening up the growth front of the colony in ■ fanlike fashion. At 25 and 50 $\mu g.ml^{-1}$, the colonies were similar to the control. On the other hand, trichorzianines A isolated from *T. harzianum* inhibited 65% of the mycelial growth of the same strain. In this case colonies were restricted but more dense than the control therefore the dry weigth was evaluated. Results showed reduction to 58% dry weigth in relation to the control.

DISCUSSION

Results showed that when trichorzianines were present in the culture media the mycelial growth of *S. rolfsii* was noticeably reduced. Moreover, at higher concentration, higher inhibition was evidenced. It has been shown previously that trichorzianines increase the permeability of synthetic lipid membranes and also cause the lysis of the *D. discoideum* amoeba (El Hajji *et al.*, 1989). Other peptidic antibiotics produced by *Trichoderma* species have shown that they act with a similar mechanism (Ramesh *et al.*, 1977). Thus, trichorzianines could have probably induced disruptions in the plasma membrane of *Sclerotium*.

Trichoderma metabolites not only affected mycelial rate ofgrowth of *S. rolfsii* but also produced ■ change in the normal morphogenetic pattern. At the highest trichorzianines concentration, the mycelium was aggregated after 10 days and grew upwards. Peptide activity was fungistatic but not fungicidic.

The differences in the inhibitory activity of TA, which is higher than TB's, could be related to the neutral property of the former and the acidic character of the latter (EL Hajji *et al.* 1987). The interaction of the peptide TB with the lipid membrane could be prevented by its acidic property (El Hajji *et al.*, 1989). The mycelial growth inhibition of *T. harzianum* by the TA seems to indicate an absence of immunity of the mycelium towards its own antibiotic metabolites contrary to the autoimmunity observed for the microcin peptide antibiotics produced by some Enterobacteriaceae (Baquero & Moreno, 1984; Gaggero *et al.*, 1993). It is assumed that in filamentous fungi the distribution of primary and secondary metabolism is separated in space and time (Moss, 1984; Griffin, 1994). In nature, metabolites such as trichorzianines are probably produced by more differenciated parts of the mycelium. They do not necessarily act on hyphal tips where growth occur. In our experiments TA was present all over the medium and by the way inhibited the growth.

Planche 1. Effet inhibiteur du mélange de peptides sur la croissance de *S. rolfsii* après 96 heures. 1: *S. rolfsii* témoin sur milieu MA; 2-4: avec mélange de peptides à diverses concentrations (25, 50, 100 $\mu g.ml^{-1}$); 5: avec la fraction à bas poids moléculaire; 6: avec le mélange de peptides après 10 jours de culture. Les cordons mycéliens se dressent en forme d'éventail. 7: Inhibition de la croissance de *S. rolfsii* par la trichorzianine A (100 $\mu g.ml^{-1}$) après 96 heures. Témoin à gauche.

We are very grateful to M. Massias (Laboratoire de Chimie, MNHN, Paris) M. Dumont (Laboratoire de Cryptogamie, MNHN, Paris) for their technical assistance.
This research has been supported by the Scientific and Technical Uruguayan-French Cooperation.

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